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Synergistic Antioxidant Activity of Milk Sphingomyeline and Its Sphingoid Base with α-Tocopherol on Fish Oil Triacylglycerol

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ABSTRACT: The effects of milk phospholipids (PLs), sphingolipids (SLs), and their sphingoid backbone on the oxidation of fish oil triacylglycerol (TAG) were examined with or without α-tocopherol. All compounds had little effect on the TAG oxidation in the absence of α-tocopherol. On the other hand, they could act synergistically with α-tocopherol. The highest synergistic activity was shown by sphingoid bases, followed by sphingomyelin (SPM) and other amine-containing PLs and SLs. This result showed that the synergistic activity increased with an increasing concentration of amine group of PLs, SLs, or sphingoid bases in the reaction mixture. The comparison of changes in α-tocopherol content in fish oil TAG and tricaprylin suggested that antioxidant compounds would be formed from the amine group and the lipid oxidation products in a mild oxidation condition controlled by α-tocopherol.

KEYWORDS: *Milk sphingomyeline, sphingoid base, synergistic effect, tocopherol, fish oil oxidation*

INTRODUCTION

Tocopherols are the most important natural antioxidants widely used for foods and cosmetics. They behave like chain-breaking electron-donor antioxidants. A tocopherol radical is stabilized by resonance and does not propagate the radical chain oxidation but forms non-radical products. Tocopherols may be more active when used in combination with other antioxidants, such as citric acid and ascorbic acid. Amine-containing glycerophospholipids (PLs), such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), have also been well-known as synergists in combination with tocopherols.[1−5] The remarkable synergistic effect of PL has been reported especially in marine lipid mixtures containing a high level of polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (20:5ω-3, EPA) and docosahexaenoic acid (22:5ω-3, DHA), although the PL had no activity on the oxidative stability of these PUFAs in the absence of tocopherols.[6−9]

The detailed mechanism responsible for the synergy of PL and tocopherols has not been well-understood. However, the amine group of PC and PE has been postulated to have an important role, such as hydrogen or electron donor, to regenerate and recycle the tocopheroxyl radical intermediate to the parent phenol, tocopherol.[10,11] Another possible role of the amine group would be a source of antioxidative pyrrole compounds formed by the non-enzymatic browning reaction with fatty acid oxidation products containing a keto group, mainly aldehydes.[12−17] The amine of PE undergoes pyrrolization 10 times more readily than that of amino acids.[17−19]

Marine PUFAs, especially, EPA and DHA, have been shown to cause significant biochemical and physiological changes in the body that most of the times result in a positive influence on human nutrition and health. On the other hand, both PUFAs are easily oxidized because of the high degree of unsaturation. Because the lipid oxidation products of EPA and DHA cause undesirable flavors and lower the nutritional quality and safety of the lipid-containing foods, prevention of the oxidation is important and essential for their application to food products. Usually, natural antioxidants, such as tocopherols, are used for this purpose; however, only tocopherol addition to the lipids rich in EPA and DHA is not always effective because of the high susceptibility of EPA and DHA to oxidation. From this viewpoint, much interest has been paid to the synergistic activity of a natural compound, such as amine−PL.[5,20] On the other hand, no study has been performed on the effect of sphingolipids (SLs), although they contain amines in their sphingosyl backbone and are one of the major lipid classes widely found in the plant and animal kingdom.

In the present study, we reported the effect of SLs on the oxidative stability of fish oil triacylglycerol (TAG) in the presence or absence of α-tocopherol. In addition, the effect of sphingoid bases on the α-tocopherol activity was analyzed. Sphingoid base is a basic structural element of SL, which is usually attaching an amide-linked long-chain fatty acid and sugar or a phosphatidylcholine on the primary hydroxyl group of the sphingoid base. The sphingoid base can be librated by the degradation of SL by chemical and biological reactions in food production and biological systems.

EXPERIMENTAL PROCEDURES

Standards and Chemicals. Sphingomyelin (SPM), lactosylceramide (LacCer), glucosylceramide (GluCer), and monosialodihexosylganglioside (GM3) were obtained from Nagara Science Co., Ltd., Oritate, Gifu, Japan. These SLs (purity > 99%) originated from bovine milk. Dihydrosphingosine (d18:0) and sphingosine (d18:1) were purchased from Avanti Polar Lipids, Inc., Alabaster, AL. *N,N*-Dimethylsphingosine (M-d18:1) was a product of Biomol International, LP, Plymouth Meeting, PA. α-Tocopherol was purchased from

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Wako Pure Chemical Ind., Ltd., Osaka, Japan. Silica gel (BW-60F) for column chromatography was purchased from Fuji Sylysia Chem., Ltd., Kasugai, Aichi, Japan. Activated carbon and celite (545 RVS) were products of Nacalai Tesque, Inc., Kyoto, Japan. Two kinds of fish oil (fish oil-1 and fish oil-2) were kindly donated from Central Research Lab., Nippon Suisan Kaisha, Ltd., Hachioji, Japan. They originated from sardine (fish oil-1) and bonito (fish oil-2). Triolein (purity > 99%) and tricaprylin (purity > 99%) were purchased from Wako Pure Chmeical Ind. and Riken Vitamin Co., Ltd., Tokyo, Japan, respectively. All of the other chemicals and solvents used in this study were of analytical grade, except that high-performance liquid chromatography (HPLC)-grade solvents were used for HPLC analysis.

Butter Serum (BS) Lipid Separation. BS was donated from Megmilk Snow Brand Co., Ltd., Saitama, Japan. The BS was extracted with 10 volumes (v/w) of ethanol and allowed to stand overnight. The solvent was filtered, and the residue was further extracted with ethanol. Both filtrates were combined and concentrated under vacuum using a rotary evaporator. The ethanol extracts were dissolved in chloroform/methanol/water (10:5:3, v/v/v). The solution was placed in a separatory funnel, shaken, and allowed to stand overnight. The lower layer was concentrated under vacuum using a rotary evaporator. The last traces of organic solvent and water were removed in a desiccator under high vacuum. The extract obtained from the BS was dissolved again in diethyl ether and allowed to stand overnight at −20 °C. After the filtration, the filter paper with the residue was washed with diethyl ether several times and the lipids (crude BS−PL) were recovered by dissolving in chloroform/methanol (2:1, v/v). On the other hand, after the concentration of the filtrate, the residue was dissolved in pyridine and allowed to stand overnight at −20 °C. After the filtration, the filtrate was concentrated and used as crude BS−SPM. Both crude BS−PL and BS−SPM were subjected to thin-layer chromatography (TLC) separation. TLC was carried out on preparative-scale silica gel plates (Merck, Darmstadt, Germany) developed with chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) or chloroform/methanol/25% ammonium solution (65:35:5, v/v/v) to obtain purified BS−PC, BS−PE, and BS−SPM.

Lipid Substrate Preparation. Two kinds of fish oils (fish oil-1 and fish oil-2) (ca. 25 g) were passed through a column (50 × 4 cm inner diameter) packed with a *n*-hexane slurry mixture of activated carbon (100 g) and celite (100 g) to remove tocopherols and pigments by eluting with *n*-hexane (1200 mL). The oil (ca. 10 g) after treatment with active carbon−celite column chromatography was further refined on a silicic acid column (50 × 4 cm inner diameter) packed with a *n*-hexane slurry of silica gel BW-60F (200 g) by eluting with *n*-hexane (200 mL) and a mixture of *n*-hexane/diethyl ether [98:2 (200 mL) and 90:10 (1200 mL), v/v]. The fraction eluted with *n*-hexane/diethyl ether (90:10) was used for the present study as fish oil TAG-1 from fish oil-1 and fish oil TAG-2 from fish oil-2, respectively.

To confirm the absence of peroxides, tocopherols, and minor lipid classes, such as free fatty acids, monoacylglycerols, and diacylglycerols in purified TAG, peroxide value (PV) analysis, HPLC analysis, and TLC analysis were carried out. The PV of the purified TAG was determined by the American Oil Chemists' Society (AOCS) Official Method.[21] An aliquot of TAG was dissolved in *n*-hexane and subjected to tocopherol analysis using HPLC. The HPLC was performed with a Hitachi HPLC system (Hitachi Seisakusho, Co., Tokyo, Japan) equipped with a pump (Hitachi L-7100) and a fluoresence detector (Hitachi L-7485). The analysis was carried out on a silica column (Develosil 100-3, 250 × 4.6 mm inner diameter, Nomura Chem. Co., Seto, Aichi, Japan) protected with a guard column (15 × 3.2 mm) with the same stationary phase. The mobile phase was *n*-hexane/2-propanol (99.2:0.8, v/v), setting the flow rate at 1.4 mL/min. To check lipid class purification of TAG, analytical TLC was carried out on a 0.25 mm silica gel plate (silica gel 60G, Merck) developed with *n*-hexane/diethyl ether/acetic acid (70:30:1, v/v/v). Lipid spot was detected with iodine vapor or 60% aqueous sulfuric acid charring. Identification of the spot was performed using standard TAG (triolein). The PV of both fish oil TAG-1 and fish oil TAG-2 was less than 0.5 mequiv/kg. HPLC and TLC analyses showed the complete removal of tocopherols and minor lipid classes from the crude lipids.

Fatty Acid Analysis of Lipids. The fatty acid composition of lipids separated from BS and of purified TAG was determined by gas chromatography (GC) after conversion of fatty acyl groups in the lipid to their methyl esters by transesterification using sodium methoxide (CH_3ONa) as the catalyst. An aliquot of lipid (ca. 10 mg) in a 10 mL sealed tube was added 2 mL of 0.5 M CH_3ONa in MeOH, and the mixture was heated to 55 °C under nitrogen in the dark. After 15 min of heating, the solution was vortexed for 1 min and then heated again for another 15 min at 55 °C. The reaction mixture was cooled and added 2 mL of *n*-hexane and 2 mL of water. The solution was shaken, and the upper hexane layer containing fatty acid methyl esters was recovered. The remaining lower layer was added by 2 mL of *n*-hexane, and the upper layer was recovered. The two parts of the upper layer obtained were combined, and the hexane solution was washed with water. The solution was evaporated to remove organic solvent and subjected to GC. The GC analysis was performed on a Shimadzu GC-14B (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame ionization detector and a capillary column (Omegawax-320; 30 m × 0.32 mm inner diameter; Supelco, Bellefonte, PA). The detector, injector, and column temperatures were 260, 250, and 200 °C, respectively. The carrier gas was helium at a flow rate of 50 kPa. The fatty acid content was expressed as a weight percentage of total fatty acids.

Oxidation and Analysis. Each 100 mg TAG sample was put in a 5 mL aluminum sealed vial with a butyl gum septum (GL Science, Tokyo, Japan) and then incubated at 37 °C in the dark. α-Tocopherol, BS lipids, standard SLs, and sphingoid bases were dissolved in a small amount of chloroform and then added to the TAG. After mixing the solution homogeneously, the chloroform was removed in vacuum, so that the α-tocopherol concentration was 0.05 wt %. The concentration of BS lipids, standard SLs, and sphingoid bases in the oxidation sample mixture was adjusted to be 1.0 wt %. In the addition of d18:0, the effect of the different concentrations (from 0.125 to 1.0 wt %) was also analyzed. Before the incubation, the level of oxygen in the headspace gas of the vial was estimated by GC (Shimadzu GC-14B).[22] GC was equipped with a thermal conductivity detector and a stainless-steel column (3 m × 3.0 mm inner diameter) packed with molecular sieve 5A (GL Science). The temperatures at the injection port, detector port, and column oven were 100, 100, and 50 °C, respectively. The helium flow was 50 kPa. More than three separate vials containing the same kind of sample were prepared and incubated. A small portion (20 μL) of the headspace gas was taken from each vial by a microsyringe through butyl gum septum at selected oxidation times. The decrease (%) in oxygen was calculated from the changes in the oxygen ratio to nitrogen compared to that before incubation. Each data value at different oxidation times of different samples was expressed as the mean ± standard deviation (SD) (n = 3−5).

Oxidation of the sample (100 mg) was also monitored by the increase in PV and the decrease in the total unoxidized PUFAs during the incubation. For the analysis, many 5 mL aluminum vials containing the same lipid sample were prepared. The vials were sealed with a butyl gum septum (GL Science) and then incubated at 37 °C in the dark. After a definite time of incubation, a vial was randomly selected from the same sample group and the lipid in the vial was subjected to PV, tocopherol, and fatty acid analyses. PV analysis was performed as described above. The change in α-tocopherol content during the oxidation was analyzed using HPLC under the conditions as described above. The amount of total unoxidized PUFAs was calculated from the ratio of total peak area of PUFAs to that of saturated fatty acids on GC. PUFAs selected for the analysis were 18:2ω-6 + 20:4ω-6 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3, while saturated fatty acids for the analysis were 14:0 + 16:0 + 18:0. GC analysis of the fatty acids was performed after conversion of the fatty acyl groups in each oxidized lipid to their methyl esters. The fatty acid methyl esters were prepared as per the method by Prevot and Mordret.[23] Briefly, to an aliquot of total lipid (ca. 10 mg), 1 mL of *n*-hexane and 0.2 mL of 2 N NaOH in methanol were added, vortexed, and incubated at 50 °C for 30 min. After the incubation, 0.2 mL of 2 N HCl in methanol solution was

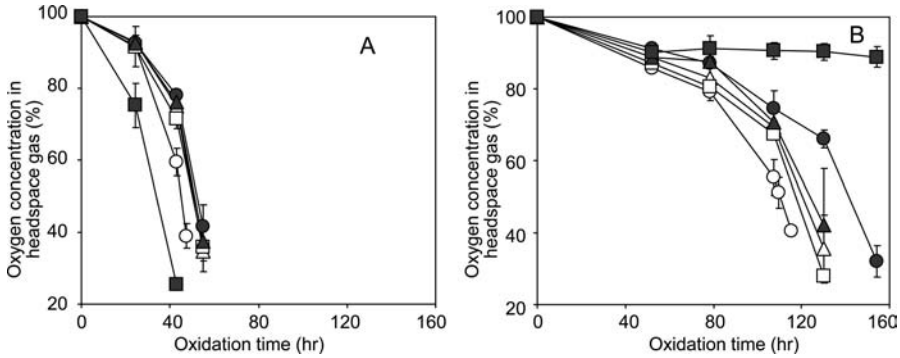

Figure 1. Effect of commercial SLs and d18:0 on the oxidative stability of fish oil TAG-1 (A) without or (B) with α-tocopherol (0.05 wt %). The data value was expressed as the mean of four separate experiments. (○) TAG alone, (△) TAG + LacCer, (□) TAG + GluCer, (●) TAG + SPM, (▲) TAG + GM3, and (■) TAG + d18:0. The concentration of SLs and d18:0 in the oxidation sample mixture was adjusted to be 1.0 wt %.

added to the solution and vortexed. The mixture was separated by centrifugation at 1000*g* for 5 min. The upper hexane layer containing fatty acid methyl esters was recovered and subjected to GC.

A larger scale oxidation was also carried out using 1 g of fish oil TAG sample containing 0.05 wt % α-tocopherol and 1.0 wt % d18:0. Tricaprylin was also used as substrate medium other than fish oil TAG in this reaction system. Oxidative stability of fish oil TAG was evaluated by the analysis of change in total PUFA content as described above. The change in α-tocoherol incubated in fish oil TAG and tricaprylin was analyzed as described above.

RESULTS

Effect of BS−PL, BS−SPM, and Commercial Milk SL on the Oxidative Stability of Fish Oil TAG. The oxidative stability of fish oil TAG-1 was analyzed in the bulk phase by measuring the decrease in oxygen in the headspace of the sample vial (Figures 1 and 2). Fish oil TAG-1 was rapidly

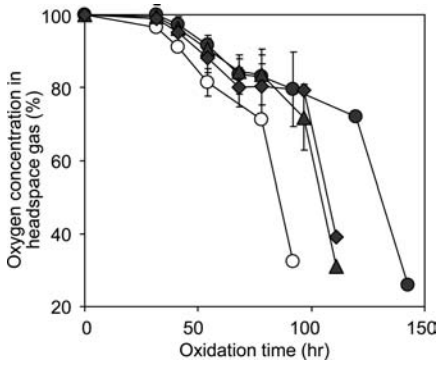

Figure 2. Effect of PC, PE, and SPM separated from BS on the oxidative stability of fish oil TAG-1 in the presence of α-tocopherol (0.05 wt %). The data value was expressed as the mean of four separate experiments. (○) TAG alone, (●) TAG + SPM, (▲) TAG + PC, and (◆) TAG + PE. The concentration of each BS lipid was adjusted to be 1.0 wt %.

oxidized, and more than 60% of oxygen in a head space of the vial was consumed by the TAG oxidation within 50 h of incubation at 37 °C in the dark (Figure 1A). This susceptibility of TAG to oxidation was due to the high level of EPA and DHA in it (Table 1). The addition of commercial SLs slightly increased the oxidative stability of the fish oil TAG (Figure 1A). The stability increased by the addition of α-tocopherol, and more than 50% of oxygen remained in the vial head space after 100 h of incubation (Figure 1B). The addition of commercial SLs originating from bovine milk greatly improved the oxidative

Table 1. Composition (wt %) of Major Fatty Acids of Substrate Lipids (Fish Oil TAG-1 and Fish Oil TAG-2) and BS−PL

fatty acid	fish oil TAG-1	fish oil TAG-2	BS−PC	BS−PE	BS−SPM
14:0	7.7	2.6	4.8	0.7	3.3
16:0	8.6	18.0	24.4	8.2	23.3
18:0		5.0	8.8	16.2	6.0
22:0			3.2	0.8	17.8
23:0			3.9		22.0
24:0			2.9		16.4
16:1ω-7	14.3	5.5			
18:1ω-9	9.1	22.0	34.1	49.2	4.4
18:1ω-7	3.3	2.9	1.9	2.1	0.3
18:2ω-6	1.4	1.3	6.8	9.7	0.8
18:4ω-3	3.4				
20:4ω-6	1.7	2.0			
20:5ω-3	23.5	4.8			
22:5ω-3	2.1	1.1			
22:6ω-3	8.6	16.0			

stability of TAG containing α-tocopherol (Figure 1B). The improvement on the oxidative stability of TAG under the presence of α-tocopherol was also found in the addition of BS−PC, BS−PE, and BS−SPM (Figure 2). When the effect of PLs and SLs was compared, SPM showed a higher activity than other SLs, such as LacCer, GluCer, and GM3 (Figure 1) or BS−PC and BS−PE (Figure 2). The effectiveness of the commercial SPM (Figure 1) was almost the same as that found in BS−SPM (Figure 2). Figure 1B also shows that fish oil TAG became much more oxidatively stable in the presence of both α-tocopherol and d18:0; however, the oxidation was promoted by the addition of d18:0 alone without α-tocopherol (Figure 1A).

Effect of Sphingoid Bases on the Oxidative Stability of Fish Oil TAG. To confirm the synergistic effect of sphingoid bases, such as d18:0, with α-tocopherol, another series of fish oil TAG oxidation was carried out (Figures 3−5). In the absence of α-tocopherol, the oxygen consumption (Figure 3A) and PV (Figure 3B) rapidly increased during the incubation. The addition of α-tocopherol delayed the increase in the oxygen consumption and PV, and the effectiveness increased remarkably in the coexistence of d18:0. Figure 4 shows the effect of the d18:0 concentration on its synergy with α-tocopherol. The synergistic effect of d18:0 increased with an increasing concentration. Other types of sphingoid bases, such as d18:1 and M-d18:1, also showed a strong synergistic effect

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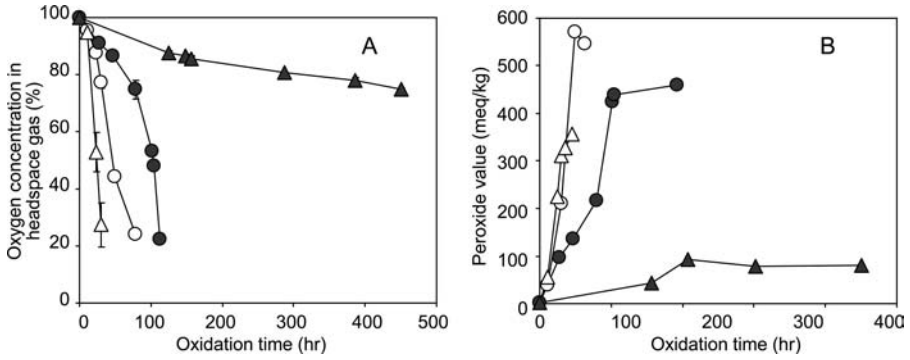

Figure 3. Effect of d18:0 (1.0 wt %) on the oxidative stability of fish oil TAG-1 without or with α-tocopherol (0.05 wt %). The data value for (A) oxygen concentration analysis was expressed as the mean of three separate experiments. For (B) PV analysis, all lipids in a sealed vial were taken at definite time incubation and subjected to the analysis. (○) TAG alone, (△) TAG + d18:0, (●) TAG + α-tocopherol, and (▲) TAG + α-tocopherol + d18:0.

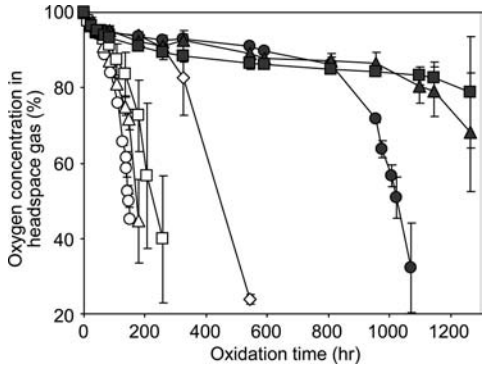

Figure 4. Effect of the d18:0 concentration on the oxidative stability of fish oil TAG-1 in the presence of α-tocopherol (0.05 wt %). The data value was expressed as the mean of four separate experiments. The concentration of d18:0 was (○) 0 wt %, (△) 0.05 wt %, (□) 0.125 wt %, (◇) 0.25 wt %, (●) 0.50 wt %, (▲) 0.75 wt %, and (■) 1.00 wt %.

with α-tocopherol (Figure 5B). The highest activity was shown by d18:0, being followed by d18:1 and M-d18:1. On the other hand, the addition of these sphingoid bases to the fish oil TAG had little effect in the absence of α-tocopherol (Figure 5A).

Decrease in α-Tocopherol Content during the Oxidation of Fish Oil TAG. In the absence of d18:0, α-tocopherol rapidly decreased during the incubation of fish oil TAG-2 (Figure 6A). After the tocopherol disappearance, the oxygen concentration in the headspace gas rapidly decreased. In the presence of d18:0, more than 60 and 80% of α-tocopherol and oxygen remained until 400 h of incubation, respectively (Figure 6A). The higher oxidative stability of the TAG containing both tocopherol and d18:0 was also confirmed by the analysis of total unoxidized PUFAs (Figure 6B). Stabilization of fish oil TAG by the addition of both α-tocopherol and d18:0 was also observed in a larger scale oxidation system composed of 1 g of sample (Figure 7). There was little decrease in the total unoxidized PUFAs during the incubation of the TAG. The tocopherol level in fish oil TAG decreased at the first stage of the incubation but, thereafter, remained to be changed a little. On the other hand, when α-tocopherol was incubated in the tricaprylin mixture, it linearly decreased.

■ DISCUSSION

Amine-containing polar lipids, such as PC and PE, have been reported to effectively inhibit the oxidation of PUFAs in the presence of α-tocopherol, while little antioxidant activity is found in these polar lipids without α-tocopherol.[1−5] Several papers demonstrated that saturated PC and PE had no synergistic effect with tocopherol,[11,24,25] while slightly oxidized PL[20] or heated PL containing PUFAs[25,26] showed the synergy with tocopherol to prevent lipid oxidation. The synergistic effect of PC and PE with α-tocopherol has also been confirmed in the present study using BS−PC and BS−PE (Figure 2). In the present study, we have also found the synergistic effect of other types of amine-containing polar lipids, SPM. The effectiveness of BS−SPM was higher than those of BS−PC

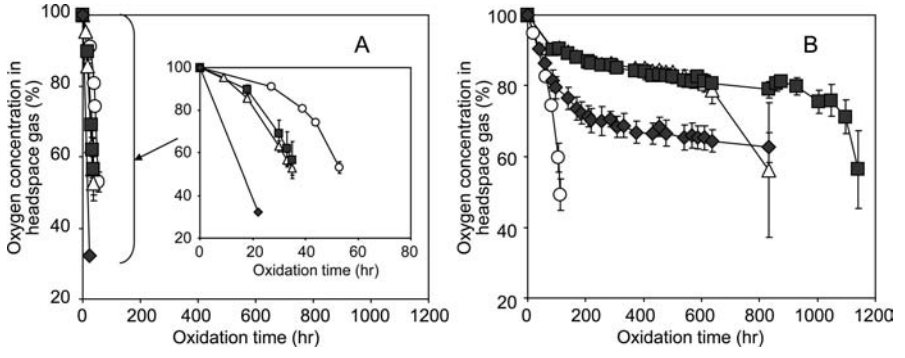

Figure 5. Effect of different sphingoid bases on the oxidative stability of fish oil TAG-1 (A) without or (B) with α-tocopherol (0.05 wt %). The data value was expressed as the mean of four separate experiments. (○) TAG alone, (△) TAG + d18:1, (■) TAG + d18:0, and (◆) TAG + M-d18:1. The concentration of each sphingoid base was adjusted to be 1.0 wt %.

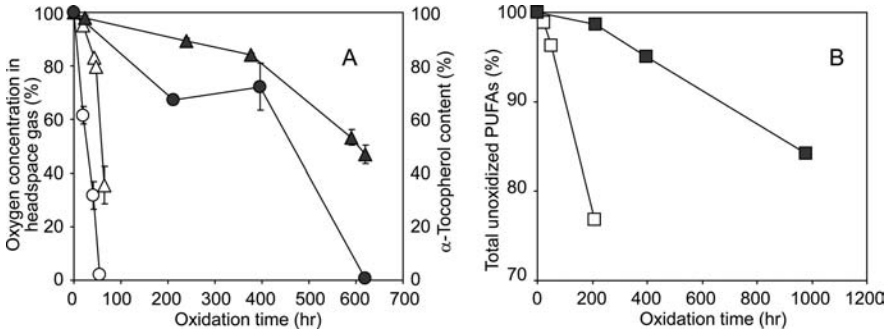

Figure 6. Effect of d18:0 (1.0 wt %) on the relationship between oxygen consumption, tocopherol content, and total content of unoxidized PUFAs during the oxidation of fish oil TAG-2 in the presence of α-tocopherol (0.05 wt %). (A) Data value was expressed as the mean of four and three separate experiments for oxygen concentration analysis and tocopherol analysis, respectively. For (B) unoxidized PUFA analysis, all lipids in a sealed vial were taken at definite time incubation and subjected to the analysis using GC. For TAG + α-tocopherol, (○) oxygen concentration, (△) tocopherol content, and (□) total unoxidized PUFAs and for TAG + α-tocopherol + d18:0, (●) oxygen concentration, (▲) tocopherol content, and (■) total unoxidized PUFAs.

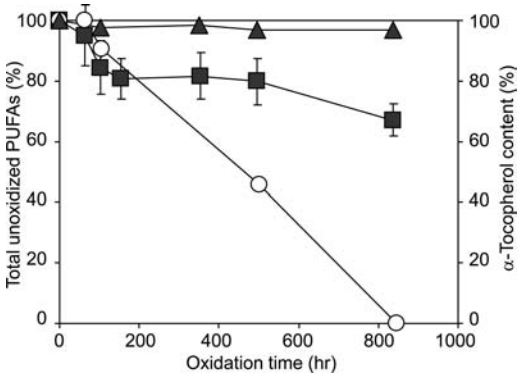

Figure 7. Decrease in the α-tocopherol content during the incubation in fish oil TAG-2 and tricaprin. The α-tocopherol content in (○) tricaprylin and (■) fish oil TAG-2 and (▲) change in the total unoxidized PUFAs during the oxidation of fish oil TAG-2.

and BS−PE (Figure 2). The higher activity of SPM might be explained by the difference in the amine group concentration of substrate lipids. As shown in Figure 8, SPM contains two amine

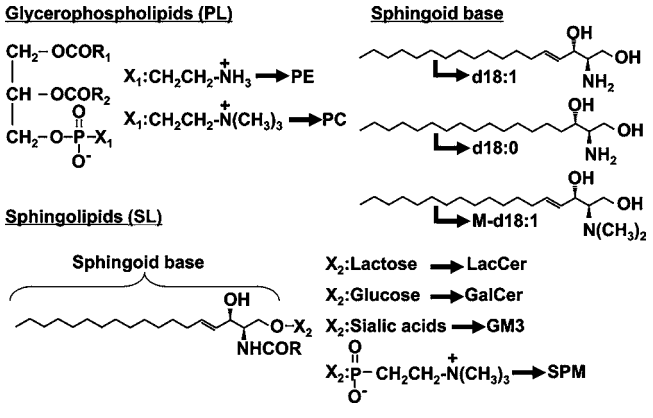

Figure 8. Chemical structures of PL and SL.

groups originated from sphingoid base and phosphocholine, while PE and PC contain only one amine group in the molecule. On the other hand, the average molecular weight (MW) of these polar lipids was almost the same. The MW of BS−PC, BS−PE, and BS−SPM could be roughly calculated to be 759.3, 721.8, and 739.7, respectively, on the basis of the MW of all fatty acyl moieties and the MW of glycerol moiety, phosphocholine, phosphoethanolamine, or sphingoid base moiety (Figure 8). The average MW of fatty acyl moieties was obtained from the weight percentage (Table 1) and MW of each PUFA. Therefore, the amine group concentration in the substrate added by 1% BS−SPM should be almost twice as much as those added by 1% BS−PC or BS−PE. This higher number of amine groups would be due to the higher synergistic activity of BS−SPM.

The synergistic effect of SPM on the antioxidant activity of α-tocopherol was confirmed using commercial milk SPM (Figure 1B). A week synergistic effect was also found in other kinds of commercial milk SLs, such as LacCer, GalCer, and GM3 (Figure 1B). An average MW of SPM, LacCer, GalCer, and GM3 varied from 750 to 930. The main reason for the higher synergistic effect of SPM would be explained by the higher number of amine groups in the SPM than those in other kinds of PLs (Figure 8). Figure 1 also shows a strong synergistic effect of sphingoid base, d18:0, with α-tocopherol. The addition of d18:0 slightly reduced the oxidative stability of fish oil TAG in the absence of α-tocopherol (Figures 1A and 3A). However, in the presence of α-tocopherol, d18:0 much more increased the TAG stability than α-tocopherol alone (Figures 1B and 3B) and the effectiveness was much stronger than that found in the addition of SPM (Figure 1B). The number of amine groups of d18:0 and SPM is 1 and 2, respectively, while the MW of d18:0 is smaller than that of SPM (Figure 8). From these structural characteristics of both compounds, the amine group concentration per TAG substrate containing d18:0 could be roughly calculated to be 1.23 times higher than that containing the same amount of SPM. This higher concentration of the amine group found in the substrate added by d18:0 might be one reason to understand the effectiveness of d18:0; however, it is difficult to explain the strong synergistic effect of d18:0 with α-tocopherol only by the different number of amine groups.

The mechanism responsible for the synergy of amine-containing polar lipids with tocopherols is not very well-understood. One possible mechanism is the involvement of the amino group of these lipids in the regeneration of tocopherol by hydrogen or electron transfer.[10,11] Another mechanism will be involvement of reaction products formed from the amine group of the polar lipids and oxidation products.[20] Hidalgo and others[12−17,27] found the browning antioxidants, kinds of pyrrole compounds, which are formed by the non-enzymatic reaction

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with the amine group of amino acids and fatty acid oxidation products, as mainly low-molecular products. They also reported the increase in the oxidative stability of vegetable oil by the addition of PE and demonstrated the formation of antioxidants from the amino group of PE and the oxidized lipid.[17,18] Therefore, the formation of antioxidants by the interaction between oxidized lipids and the amine group of polar lipids might be a reason for the increase in the oxidative stability of fish oil TAG added by PL and SPM. However, no antioxidant activity was found in the addition of PL and SPM to fish oil TAG without α-tocopherol (Figures 1A, 3, and 5A). The presence of α-tocopherol was essential for amine-containing polar lipids to show antioxidant activity (Figures 1B, 3, and 5B).

As shown in Figure 7, in a larger scale oxidation of fish oil TAG, α-tocopherol decreased during the first 100 h of incubation, but thereafter, a little change in both α-tocopherol and total unoxidized PUFAs was observed. This result suggests the formation of antioxidant compounds during the first stage of the incubation. These antioxidant compounds may prevent effectively the oxidations of PUFAs in fish oil TAG and of α-tocopherol. On the other hand, the α-tocopherol content in tricaprylin linearly decreased and disappeared after 800 h of incubation, although the tricaprylin substrate mixture contained the same amount of d18:0 and α-tocopherol as that found in fish oil TAG. This result indicates that only d18:0 could not produce antioxidants to inhibit the α-tocopherol oxidation. For the formation of antioxidant compounds, oxidation products as seen in fish oil TAG oxidation would be needed.

As Hidalgo and others[12−17,27] have reported, several kinds of reaction products would be formed from the amine group of polar lipids and oxidation products from fish oil TAG. These products showed antioxidant activity only in the presence of amine-containing polar lipids, α-tocopherol, and oxidized lipids in the present study. However, two combinations of amine-containing polar lipids + α-tocopherol (Figure 7) or amine-containing polar lipids + oxidized lipids (Figures 1, 3, and 5) were not enough to show antioxidant activity. Therefore, the formation of antioxidant compounds from the amine-containing polar lipids and oxidation products might require the presence of α-tocopherol. Mild oxidation conditions controlled by α-tocopherol may be important for the formation of the antioxidant compounds. Although the structures of the antioxidant compounds have not been made clear, they could effectively inhibit the fish oil TAG oxidation with the following mechanisms: (a) regeneration of α-tocopherol to inhibit the lipid oxidation, (b) direct inhibition of lipid oxidation, and (c) a and b.

Different sphingoid bases gave different effectiveness in the synergistic activity (Figure 5). Two sphingoid bases containing primary amine structure (d18:0 and d18:1) showed higher activity than M-d18:1 containing tertiary amine structure (Figure 8). The same effect of difference in the amine structure has been found in PL having different organic bases, primary amine (PE) and tertiary amine (PC). Kashima et al.[4] have reported that the oxidative stability of perilla oil increased more markedly by the addition of PE than that of α-tocopherol alone, while PC had no synergistic effect. The higher synergistic effect of PE than that of PC has been reported by other researchers.[9,11,28] These results may suggest the preference of the primary amine structure for the reaction with lipid oxidation products compared to the tertiary amine structure.

The oxygen absorption level, PVs, and a decreased level of PUFA observed in the present study were extremely high. These levels were generally higher than those expected for food lipids. In addition, volatile compound analysis will be most important to evaluate the lipid quality for food use. From the present model study, a combination of sphingoid base and α-tocopherol has been suggested to be effective in the inhibition of fish oil oxidation. Thus, a further study will be required to measure the effect of sphingoid base with α-tocopherol on a lower lipid oxidation level, especially focusing on volatile compound formation.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BS, butter serum; d18:0, dihydrosphingosine; d18:1, sphingosine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC, gas chromatography; GluCer, glucosylceramide; GM3, monosialodihexosylganglioside; HPLC, high-performance liquid chromatography; LacCer, lactosylceramide; M-d18:1, *N*,*N*-dimethylsphingosine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, glycerophospholipid; PUFA, polyunsaturated fatty acid; PV, peroxide value; SL, sphingolipid; SPM, sphingomyelin; TAG, triacylglycerol; TLC, thin-layer chromatography

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